



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Ronald B. Gartenhaus

Examiner: Hong Sang

Serial No.: 09/709,131

Art Unit: 1643

Filed: November 10, 2000

For: MCT-1, A Human Oncogene

37 C.F.R. 1.132 Declaration

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Dr. Leo I. Gordon, declares that:

1. I am currently the Abby and John Friend Professor of Cancer Research and Professor of Medicine, Northwestern University Medical School; I am currently the co-chair of the Eastern Cooperative Oncology Group (ECOG) lymphoma committee and have been in that position since 1998; I was Chief of the Division of Hematology/Oncology and Associate Director for Clinical Sciences at the Robert H. Lurie Comprehensive Cancer Center of Northwestern University from 1996-2006; I was an ad hoc member of the Clinical Oncology Study Section in the Center for Scientific Review at the National Cancer Institute from 2001-2004 a permanent member of this Study Section from 2004-2008 and Chair of the Study Section from 2006-2008.

2. I have known Dr. Ronald Gartenhaus, the inventor on the above-referenced patent application, since 1999 when he became a faculty member at Northwestern University Medical School in the Division of Hematology/Oncology.

3. Dr. Gartenhaus was hired as a faculty member at Northwestern University in part because of his work on the MCT-1 protein. I was familiar with his work from his article entitled "*A novel candidate oncogene, MCT-1, is involved in cell cycle progression*" (Prosniak M, et al. Cancer Res. (1998) Vol. 1, pp 4233-7). This article described MCT-1 protein as having properties that would have made it likely to be involved in cell cycle regulation pathways that were known at the time to be involved in malignancies. These properties included:

- A predicted structural homology between MCT-1 protein and cyclin, the latter protein being known to participate in cell cycle regulation through pathways involving the tumor suppressor protein p53;
- MCT-1 overexpression decreases duration of the G1/S phase of the cell cycle; and
- MCT-1 has transforming ability in vitro.

Therefore, the publication showed that MCT-1 protein participates in cell cycle regulation pathways, and in my opinion strongly suggested that MCT-1 was involved in unregulated cell proliferation of the type common to many malignancies. In view of this, a utility for antibodies specific for the MCT-1 protein was evident to me from Dr. Gartenhaus' article. For example, it was evident to me that antibodies to MCT-1 protein could have been useful for analyzing MCT-1 protein expression and localization in cells that exhibited dys-regulated growth, for determining whether MCT-1 mRNA expression was correlated with protein expression, and for evaluating compounds for effects on the expression of MCT-1 protein, which could have therapeutic applications.

From a review of the above referenced patent application, it appears that essentially the same data that is presented in Dr. Gartenhaus' article is included in the patent application. Therefore, it is my opinion that uses for antibodies that were evident to me from the article would have also been evident to me from the patent application.

4. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

Respectfully submitted,

12-4-08

Date

A handwritten signature in cursive script, appearing to read "Leo I. Gordon", is written over a horizontal line.

Dr. Leo I. Gordon

A Novel Candidate Oncogene, *MCT-1*, Is Involved in Cell Cycle Progression

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Abstract

Using the arbitrarily primed-PCR (AP-PCR) assay to detect genetic abnormalities that occur in a panel of lymphoid cell lines, we identified an amplified stretch of genomic DNA that contained a putative open reading frame. Northern blot analysis with this genomic clone revealed widespread low level expression in normal human tissue. The full cDNA sequence was obtained with no significant homology to any known genes in the genome database. We termed this novel gene with multiple copies in a T-cell malignancy as *MCT-1*. *MCT-1* was localized to the long arm of chromosome Xq22-24 by fluorescence *in situ* hybridization analysis. Although there was no significant homology at the primary sequence level, there was a limited degree of amino acid homology with a domain of cyclin H that appears to specify protein-protein complexes. This relationship between *MCT-1* and cyclin H implied a potential role for *MCT-1* in cell cycle regulation. Overexpression of *MCT-1* increased the proliferative rate of cells by decreasing the length of the G₁ phase without a reciprocal increase in the S and G₂-M phases. Recent work has established the role of cell cycle regulatory molecules in the development of certain human malignancies. Therefore, we investigated the transforming ability of *MCT-1* overexpression using soft agar growth assays and demonstrated that only *MCT-1*-overexpressing cells were able to establish colonies. Taken together, *MCT-1* is a novel candidate oncogene with homology to a protein-protein binding domain of cyclin H.

Introduction

Most cancers have both random and nonrandom chromosomal abnormalities (1). Single copy losses and gains have been well characterized in many malignancies (2). Many of these nonrandom chromosomal abnormalities have been invaluable in identifying those genes involved in tumor formation. Gene amplification is often associated with oncogenesis and drug resistance (1). The contribution of oncogene amplification to lymphoid neoplasms has not been well established (3). A PCR-based method for detecting genetic lesions is the AP-PCR² assay (4-6). Different genomic DNA templates will generate electrophoretic patterns, referred to as DNA fingerprints, with a number of differences proportional to the degree of dissimilarity between the two genomes. Additionally, it is possible to detect losses or gains in the number of copies of a target genomic sequence by the differences in the intensity of the corresponding band. Because the AP-PCR method is quantitative as well as qualitative, it can also identify amplified regions of DNA as well. In the present study, we screened a panel of T-cell leukemia/lymphoma lines using AP-PCR and identified an amplified DNA sequence that encodes a novel gene with limited amino acid homology to a region of cyclin H that appears

to specify protein-protein interactions. We termed this novel gene with multiple copies in a T-cell malignancy as *MCT-1*. Recent work has demonstrated that cell cycle regulatory molecules can participate in oncogenesis through amplification and overexpression (7-12). Our data demonstrate a shortened G₁ phase, decreased cell doubling time, and anchorage-independent growth in *MCT-1*-overexpressing cells, further supporting this important association of cell cycle regulatory molecules and tumorigenesis.

Materials and Methods

AP-PCR. Genomic DNA was prepared from all T-cell lines and normal PBL samples. All reactions were carried out in a 25- μ l volume containing 10 mM Tris-HCl (pH 8.3), 200 mM each deoxynucleotide triphosphates, 50 mM KCl, 5 mM MgCl₂, 25 pmol of 10-mer arbitrary primer, 0.1 mg of DNA template, and 1 unit of Taq DNA polymerase (Fisher Biotech). A panel of 10-20-mer primers were labeled with T4 polynucleotide kinase and [γ -³²P]ATP. All reactions were performed with a GeneAmp PCR System 9600 (Perkin-Elmer). The profile was as follows. The first five cycles of the temperature profile: denaturation at 95°C for 30 s, primer annealing at 25°C for 1 min, and extension for 1 min at 72°C. The last 25 cycles were: denaturation at 95°C for 30 s, primer annealing at 30°C for 30 s, and extension for 1 min at 72°C. PCR products were separated by electrophoresis in denaturing 8 M urea/polyacrylamide gels, followed by autoradiography.

Cloning and Sequencing of Genomic *MCT-1* Sequences Amplified by AP-PCR. The band that appeared to be amplified in the HUT 78 lane relative to the corresponding bands from other cell lines and normal lymphocytes was isolated for cloning and sequencing. This band was excised from gels and incubated in 10 ml 1 \times Assay Buffer A (Fisher Biotech) at 90°C for 10 min. Five μ l of eluted DNA was reamplified with the same AP-PCR primer as before with MgCl₂ concentration of 5 mM for 30 cycles at 30°C. The PCR product was analyzed in polyacrylamide gel to confirm its size and purity. The amplified DNA was cloned into compatible thymidine pMOSBlue T-vector (Amersham). The presence of an appropriate insert was determined using direct colony PCR with T7- and U19-mer pMOSBlue-specific primers. Sequencing was performed using established methodology. Sequences obtained from several clones were compared with known sequences in the GenBank database using the BLASTn and BLASTx program (13).

Isolation and Sequencing of *MCT-1* cDNA. The full-length *MCT-1* cDNA sequence was obtained by the RACE method as described previously (14). Briefly, 5' and 3' RACE PCRs were carried out using total RNA obtained from normal human PBLs. In the 3' RACE procedure, first-strand cDNA synthesis was initiated using a poly(T) adapter primer. The cDNA was amplified by PCR with the GSP (5'-GATCCTGTCAAAATAGTCCGATGC-3'), and the abridged universal amplification primer was supplied by the manufacturer (Life Technologies, Inc.), which is homologous to the adapter sequence used to prime first cDNA synthesis. In the 5' RACE protocol, first-strand cDNA was synthesized using GSP1 (5'-TCCAGGAGAAGTTAAGCCTG-3'). After adding a homopolymeric tail to the 3'-end of the cDNA, amplification was performed using a nested, GSP2 (5'-GACAGGATCTTTCTTAGGCATG-3') that anneals to a site located within the cDNA molecule and a novel deoxyinosine containing anchor primer supplied by the manufacturer (Life Technologies, Inc.). PCR reaction conditions were carried out as specified by the manufacturer (Life Technologies, Inc.). GSPs were designed based on sequence data obtained from the original AP-PCR amplified fragment containing a putative open reading frame.

Plasmid Construction, Cell Transfection, and Cell Culture. The full length *MCT-1* cDNA expression vector (pCMV-*MCT-1*) was made using

Received 6/26/98; accepted 8/11/98.

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² The abbreviations used are: AP-PCR, arbitrarily-primed PCR; PBL, peripheral blood lymphocyte; RACE, rapid amplification of cDNA ends; GSP, gene-specific primer; CMV, cytomegalovirus.

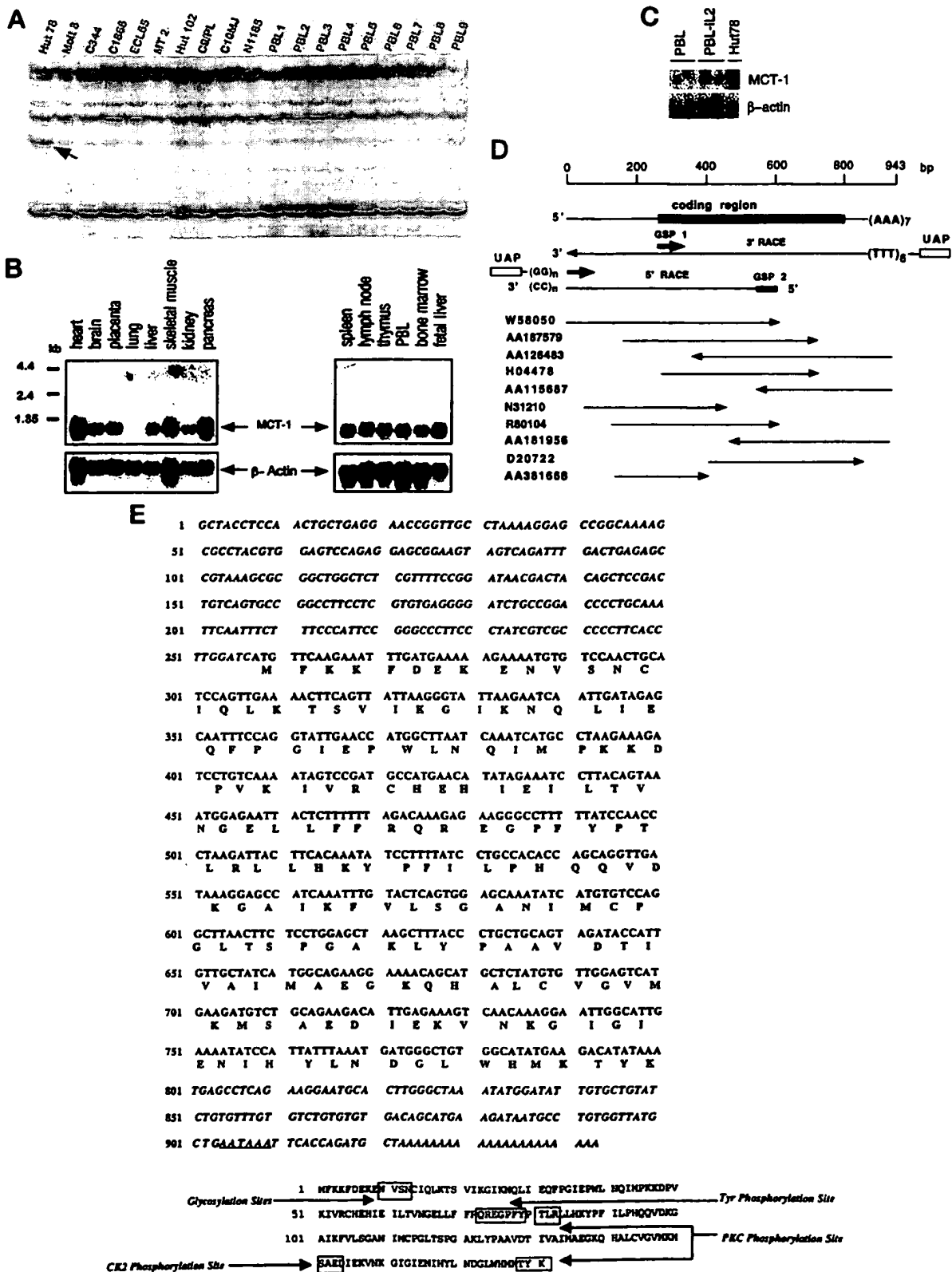


Fig. 1. Isolation and characterization of *MCT-1* cDNA. **A**, AP-PCR analysis of 10 T-cell tumor cell lines and 9 normal donor PBLs identified an amplified band in the HUT 78 cell line that was directly cloned into pMOSBlue vector for sequence analysis. This amplification was confirmed by Southern blot analysis (data not shown). **B**, Northern blot analysis (2 μ g of polyA-selected RNA) of human multiple tissue blots with the genomic probe demonstrating low level ubiquitous expression of *MCT-1* relative to β -actin. Similar results were obtained with a 5' cDNA probe (data not shown). **C**, Northern blot analysis (10 μ g of total RNA) of HUT 78 and PBL \pm interleukin 2 revealing elevated levels of *MCT-1* in HUT 78 compared with both stimulated and nonstimulated normal lymphocytes. **D**, alignment of cloned cDNA and expressed sequence tags. Below our novel mRNA are the 5' and 3' RACE cDNA fragments with the GSPs noted by closed boxes. Arrows, the positions, relative lengths, and orientations of overlapping expressed sequence tags. On the left are the GenBank accession numbers. **E**, nucleotide and deduced amino acid sequence of *MCT-1*. The full-length *MCT-1* cDNA encodes a predicted polypeptide of 181 amino acids. The polyadenylation signal is underlined. Five putative posttranslational sites predicted by MotifFinder are indicated by arrows.

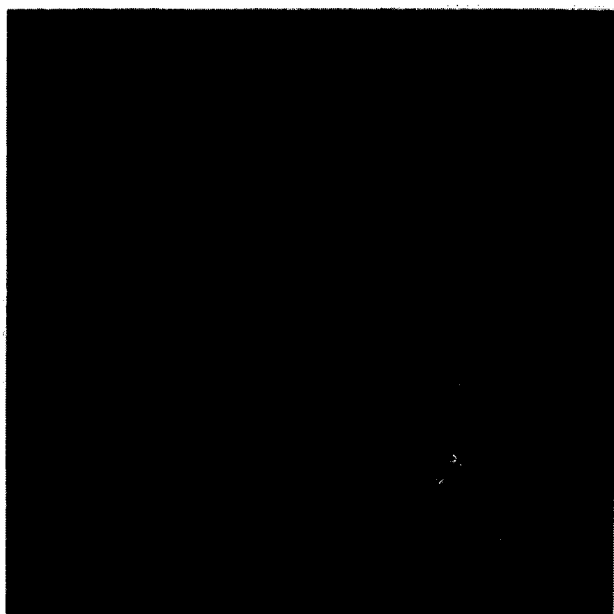


Fig. 2. Chromosomal localization of *MCT-1* was ascertained by fluorescence *in situ* hybridization analysis. Briefly, a BAC library was screened using specific 5' and 3' PCR primers from our full-length cDNA sequence. Two clones (BAC 5839 and BAC 5841) were positive for both 5' and 3' primers. Each BAC probe was labeled with digoxigenin dUTP by nick translation. The labeled probe was hybridized to normal metaphase chromosomes from phytohemagglutinin-stimulated PBLs. Specific hybridization signals (red) were observed on the long arm of X chromosome (Xq22-24) using BAC 5839 in all cells examined. A centromeric probe of the X chromosome was simultaneously hybridized (green). No signal was detected on any other chromosome using these probes.

specific restriction enzymes. To generate pCMV-*MCT-1*, the pCMV vector containing the T7 promoter linked to the coding sequence of *MCT-1* was generated using (+) 5'-GCTGAGGATCCGGTTCCTAAAAG-3' and (-) 5'-TCTGGTGAATTCATTCAGCATAA-3' primers to amplify PBL cDNA, then digested with *Bam*HI and *Eco*RI, and ligated to pCMV. This construct was verified by DNA sequence analysis. NIH 3T3 cells were transfected using the Lipofectamine method according to the suppliers instructions (Life Technologies, Inc.). Transfectants were selected after growing in 10% serum DMEM supplemented with 1000 μ g/ml geneticin (G418) for 2 weeks. Individual clones of transfected cells were obtained by limiting dilution.

Flow Cytometric Analysis. After serum depletion for 48 h, cells were replated at 3×10^5 cells per 100-mm dish in DMEM plus 10% FCS. Every 1–2 h, cells were collected and analyzed for DNA content by flow cytometry. Cells were trypsinized, fixed, and stained with propidium iodide with 0.6% NP40 and 2 mg/ml RNase. Fluorescence data were collected with the Coulter Epics XL-MCL flow cytometer, and the percentage of cells within the G_1 , S, and G_2 -M phases of the cell cycle was determined by analysis with the software program MultiCycle (Phoenix).

Northern Blot Analysis. Both a genomic and 5' cDNA *MCT-1* probe were random primed with PRIME-IT kit (Stratagene) and purified using PrimeErase Quik columns (Stratagene) according to the supplier's directions. Human multiple tissue blots (2 μ g of polyA-selected RNA) were then hybridized with either probe according to the supplier's directions (Clontech). HUT 78 and normal PBLs (10 μ g of total RNA) were analyzed in a similar fashion. Quantification was carried out using the STORM PhosphorImager 860 (Molecular Dynamics, Sunnyvale, CA).

Transformation Assays. Soft agar cultures were carried out essentially as published (15). Briefly, NIH 3T3 fibroblast monolayers, transfected with vector alone pcDNA3 (pCMV) or full-length *MCT-1* cDNA expression vector (pCMV-*MCT-1*) after a 2-week selection in G418-supplemented media, were seeded in 0.3% Bacto-Agar suspension supplemented with DMEM plus 20% FCS. This suspension was overlaid above a layer of 0.5% agar in the same medium on a 90-mm plate. All samples were set up in triplicate. The cells were refed every 4 days. After 4 weeks, soft agar-grown colonies were assessed both by the naked eye and by microscopy after incubating cells for 24 h with 0.5 mg/ml p-Iodonitrotetrazolium violet.

Results and Discussion

We screened a panel of T-cell leukemia/lymphoma lines using AP-PCR (4–6) and identified an amplified DNA sequence subsequently confirmed by Southern blot (Fig. 1A) in the Hut 78 cell line derived from a patient with Sezary syndrome (16). Sequence analysis of the amplified genomic fragment revealed a putative exon (orf), and we were able to amplify a similarly sized fragment from cDNA in the Hut 78 cell line with a reverse transcription-PCR assay (Data not shown). This genomic probe was then used for expression analysis of a variety of normal human tissues, revealing low level ubiquitous expression by Northern blotting of a single transcript running just below 1 kb (Fig. 1B). Expression of *MCT-1* in HUT 78 was compared with normal PBLs (\pm interleukin 2 stimulation), revealing increased levels only in the HUT 78 cell line (Fig. 1C).

To further characterize the novel expressed gene sequence, we obtained a full-length cDNA sequence by the RACE method (14) and found an open reading frame that encodes a protein of 181 amino acids with a predicted molecular mass of 20 kDa (Fig. 1E). Using the MotifFinder program, five posttranslation modification sites were found in the predicted polypeptide (Fig. 1E). No significant homology to known genes in the genome database was identified using the BLAST program (13); however, searching dbEST, we found several overlapping expressed sequence tags that had homology with our cDNA sequence (Fig. 1D). *MCT-1* was localized to the long arm of chromosome Xq22–24 using Bac clones positive for both 5' and 3' regions of our full length *MCT-1* cDNA (Fig. 2).

Although there was no significant homology at the primary sequence level using the BLAST program, there were interesting alignments at the structural protein level (NRL-3D Database, Amino Acid Sequence Extraction, Brookhaven Structural Database). One potentially important region was at the NH_2 -terminal half that revealed a sequence identity of 32% over a 58-amino acid stretch with the $COOH$ -terminal domain of cyclin H (Fig. 3). This region of cyclin H appears to specify protein-protein complexes (17). The statistical significance of the sequence alignment was assessed using a Monte Carlo statistical method (random quality, $N = 100$, of 26.4 ± 4.4 and an aligned quality of 48.0; Refs. 18 and 19). The gaps observed within this alignment correspond to regions of random coil (regions that can easily accommodate insertions and deletions) within the cyclin H molecule and do not violate the structural integrity of the hydrophobic core. This relationship between *MCT-1* and cyclin H implied a potential role for *MCT-1* in cell cycle regulation. To explore this hypothesis, we examined the effects of *MCT-1* overexpression on cell growth and cell cycle progression in NIH 3T3 fibroblasts by flow cytometric analysis on synchronously growing cell cultures. After transfection of the full-length construct, a marked decrease in cell doubling time was observed (Fig. 4a) accompanied by a significant decrease in the duration of the G_1 -S phase of the cell cycle compared with controls (Fig. 4b). Overexpression of *MCT-1* increases the proliferative rate of cells by decreasing the length of the G_1 phase without a reciprocal increase in the S and G_2 -M phases. Because *MCT-1*

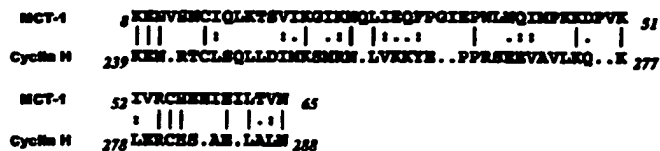
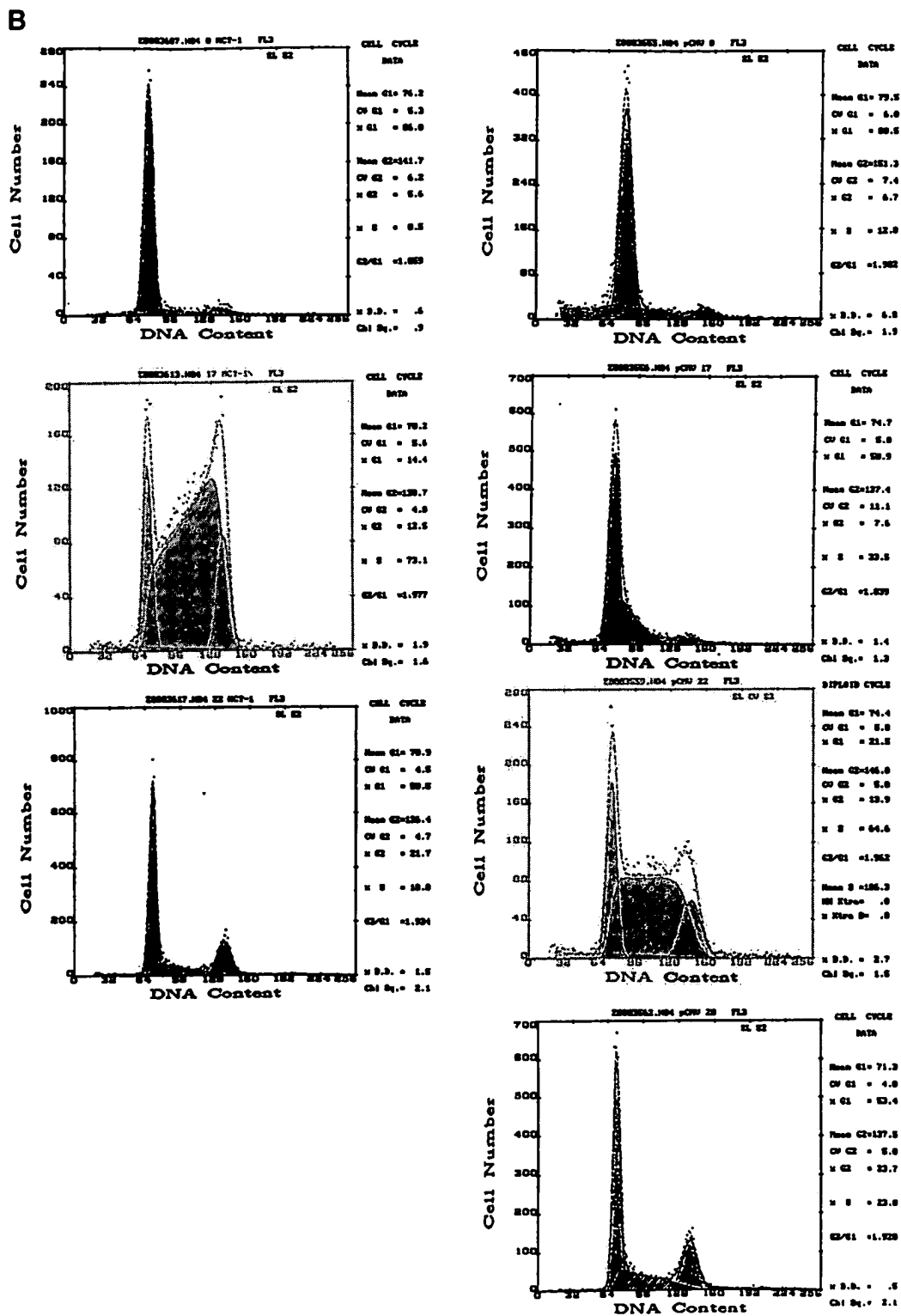
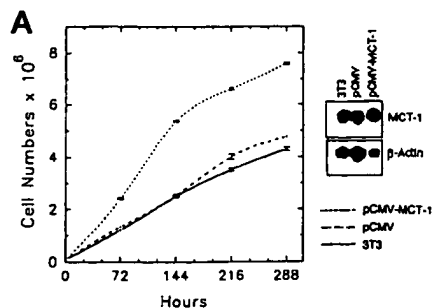


Fig. 3. Alignment at the protein level (NRL-3D Database, Amino Acid Sequence Extraction, Brookhaven Structural Database) of *MCT-1* with cyclin H. One potentially important region was at the NH_2 -terminal half that revealed a sequence identity of 32% over a 58-amino acid stretch with the $COOH$ -terminal domain of cyclin H (see text for details). This region of cyclin H appears to specify protein-protein complexes.



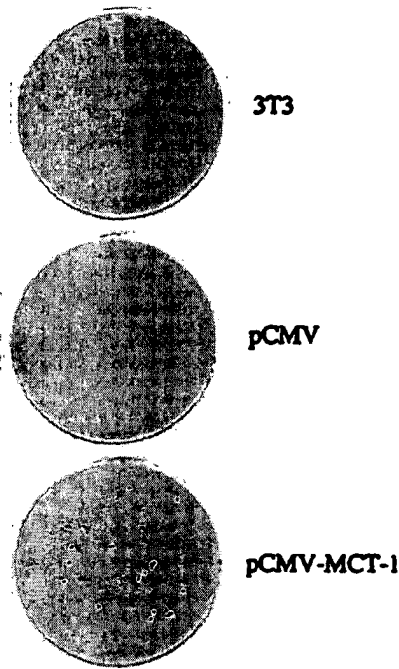


Fig. 5. *MCT-1* has transforming ability *in vitro*. A soft agar transformation assay is shown. Briefly, NIH 3T3 fibroblast monolayers transfected with vector alone pCDNA3 (pCMV) or full-length *MCT-1* cDNA expression vector (pCMV-*MCT-1*) were seeded in 0.3% Bacto-Agar suspension supplemented with DMEM plus 20% FCS. This suspension was overlaid above a layer of 0.5% agar in the same medium on a 90-mm plate. All transfections were set up in triplicate. The cells were refed every 4 days. Colonies were scored both by the naked eye and by microscopy. A representative experiment demonstrates no growth in pCMV vector-transfected cells or the parent NIH3T3 cell line; however, in pCMV-*MCT-1*-transfected cells, there were numerous colonies after 4 weeks.

overexpression demonstrated a marked effect on cell proliferation and cell cycle progression, we decided to examine whether *MCT-1* could induce NIH 3T3 transformation. The ability of *MCT-1* overexpression to promote anchorage-independent growth was assessed by soft agar growth assays and demonstrated that only *MCT-1*-overexpressing cells were able to establish viable colonies (Fig. 5).

Although the precise biological function of *MCT-1* is still unknown at present, several observations suggest a cell cycle regulatory role for *MCT-1*. These observations include the significant decrease in cell doubling time and shortening of the G_1 phase of the cell cycle in *MCT-1*-overexpressing cells. Moreover, phosphorylation is a common mechanism for regulating cell cycle-related proteins, and the *MCT-1*

Fig. 4. (opposite page) Effect of *MCT-1* overexpression in NIH 3T3 fibroblasts on cell proliferation and cell cycle progression. Stable cell lines overexpressing human *MCT-1* were established by transfecting NIH 3T3 fibroblasts with either vector alone pCDNA3 (pCMV) or full-length *MCT-1* cDNA expression vector (pCMV-*MCT-1*), both conferring G418 resistance. In A, transfected cells were grown in selection medium for 2 weeks (DMEM complete plus 1000 μ g/ml G418; mock-transfected cells had 100% cell death). Controls included cells transfected with vector (pCMV) alone and untransfected 3T3. Population doubling times were calculated by counting cells every 72 h for 12 days. Data are presented as mean ($n = 3$ for each data point); bars, SD. Note increased level of *MCT-1* RNA in 3T3 cells transfected with pCMV-*MCT-1* expression vector relative to controls. B, after serum depletion for 48 h cells were replated at 3×10^5 cells per 10-cm dish in DMEM plus 10% FCS. Every 1–2 h, cells were collected and analyzed for DNA content by flow cytometry. Fluorescence data were collected with the Epics Coulter flow cytometer, and the percentage of cells within the G_1 , S, and G_2 -M phases of the cell cycle were determined by analysis with the software program MultiCycle (Phoenix). Left, histograms of pCMV-*MCT-1*-transfected cells at 0, 17, and 22 h. Right, histograms of pCMV-transfected cells at 0, 17, 22, and 28 h. At 17 h after release from G_1 arrest, the vector-transfected cells are predominantly in G_1 ; however, the majority of pCMV-*MCT-1*-transfected cells are already into the S phase of the cell cycle. At 22 h, the majority of pCMV-*MCT-1*-transfected cells have returned to G_1 , in contrast to the pCMV-transfected cells, which do not return to G_1 until 28 h. Parental 3T3 cell lines gave similar results as the pCMV-transfected cells (data not shown). Similar results were obtained in four replicate experiments.

protein is predicted to have at least four phosphorylation sites, including tyrosine, CK2, and PKC phosphorylation sites. Finally, there is also a stretch of 58 amino acids at the *MCT-1* NH_2 terminus with homology to the protein-protein interacting domain of cyclin H.

The long arm of chromosome X has been found to contain amplified DNA regions in a variety of lymphoid neoplasms using comparative genomic hybridization (20, 21). Because *MCT-1* was localized to chromosomal bands Xq22–24, we analyzed primary samples from patients with two lymphoid malignancies, Cutaneous T-cell Lymphoma ($n = 40$) and Chronic Lymphocytic Leukemia ($n = 20$) but found that they did not exhibit genomic amplification of *MCT-1* (data not shown). However, it does appear that *MCT-1* overexpression contributes to deregulated cell cycle progression and proliferation *in vitro*. Further support for a role of *MCT-1* as a putative oncogene comes from its ability to support soft agar growth in fibroblasts overexpressing *MCT-1*. Ongoing studies examining the interaction of *MCT-1* with other molecules, including regulatory components of the G_1 -S phase of the cell cycle, will help elucidate the mechanisms through which it accelerates cell cycle progression. Finally, evaluating the amplification and expression of *MCT-1* in a range of human tumors will further delineate its contribution to tumor development.

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